

METHOD FOR PRODUCING A RECOMBINANT PROTEIN USING POLLEN

TECHNICAL FIELD

5 The present invention relates to a method for producing recombinant protein using pollen. More particularly, the present invention relates to a method for producing recombinant protein coding a target gene by introducing the target gene into plant pollen by following transformation.

10 BACKGROUND ART

 On attempts to produce recombinant protein in transgenic plant systems, much research has been conducted on the production of protein through intracellular accumulation (cytoplasm or intracellular organelle) in specific tissue or organ (seed, tuber, etc.) by employing plant, plant organs, cultured plant cells
15 or secretion systems (intracellular gap, secretion in medium). (Molony, *Biotechnol. Eng.*, 9, 3, 1995; Kusnadi et al., *Biotechnol. Bioeng.*, 56, 473, 1997; Smith & Glick, *Biotechnol. Adv.*, 18, 85, 2000; Sijmons et al., *Bio/Technology*, 8, 217, 1990; Doran, *Curr. Opin. Biotechnol.*, 11, 199, 2000; Boothe et al., *Drug Develop. Res.*, 42, 171, 1997; Giddings et al., *Nature Biotechnol.*, 18, 1151,
20 2000).

 Recombinant protein synthesizing systems that employ plants provide many advantages over other recombinant protein synthesizing systems employing animal cells or bacteria, such as the moderate cost of cultivation control, feasibility of scale-up, similarity to human glycosylation or folding,
25 absence of product impurities from transgenic animals or bacteria, and absence of contaminations of human-derived proteins.

 Recombinant protein production systems that employ transgenic plant cell lines are targeted to produce valuable proteins within a short period in

bioreactors. This requires careful control and analysis techniques to operate the bioreactors for the maintenance and proliferation of transgenic plant cell lines.

Regarding an invention that employs plant pollen, US patent No. 5,929,300 describes a method for producing transgenic plants by introducing a gene into the plant pollen using *Agrobacterium*, pollinating with the pistil, obtaining the seeds, and then germinating them to obtain the resulting transformed plant. However, it encountered problems with the long expression period of the target gene from obtaining the seeds from the transformation and inbreeding and because of the complicated process. In addition, only the pollen of annual plants, including tobacco and cotton, could be used, except for the pollen of trees such as fine, ginkgo tree, etc.

In studying to solve the said problems, the inventor demonstrated that when recombinant gene is introduced into plant pollen via *Agrobacterium tumefaciens* and vacuum infiltration and then pollen tube growth is induced, it could result in the mass production of recombinant protein within a short period.

Up to now, there are no cases that employ plant pollen as the host for producing recombinant protein. However, the following merits can be expected when pollen is used to produce recombinant protein:

First, a production system with pollen cells can be utilized at a lower price compared to production systems with cell lines because pollen cells can be collected plentifully at lower prices and there are no complicated costs from cell proliferation, maintenance of cellular genetic stability and operation of bioreactors.

Second, the gene can be easily introduced via the method of particle bombardment, vacuum infiltration and electroporation in a production system employing pollen cells (Tjokrokusumo et al., *Plant Cell reports*, 19, 792, 2000; Fernando et al., *Plant Cell reports*, 10, 224, 2000).

Third, recombinant protein can be directly produced from pollen growth

culture from a few times to a maximum of several days in a simple liquid medium supplemented with 3~4 kinds of chemicals, including sugar.

Fourth, plant pollen is a very excellent protein-producing host with timely and economical efficiency, since the recombinant protein can be collected
5 in a simple medium according to the use of secretion signals of various kinds of protein secreted by the pollen.

The inventor accomplished the present invention through genetic introduction and expression by recognizing that plant pollen is a producing host of recombinant protein that can produce protein in a short period at lower prices.

10

DISCLOSURE OF THE INVENTION

It is, therefore, an object of the present invention is to provide a method for producing recombinant protein using plant pollen as a producing host of various kinds of recombinant protein for use in testing, diagnosis and prevention
15 and for industrial use.

The above object of the present invention is achieved by collecting plant pollen, culturing them, introducing a target gene into the pollen via vacuum infiltration, and expressing and identifying recombinant protein from the germinated pollen.

20 The process of the present invention consists of isolating a pollen from the plant, culturing the pollen in a pollen growth medium, inserting a target gene into an *Agrobacterium* vector and then transforming the *Agrobacterium*, infecting the transformed *Agrobacterium* into the cultured plant pollen, and growing the pollen with the target gene to obtain the resulting recombinant protein.

25 The present invention's method of producing recombinant protein using pollen will be explained in greater detail below:

STEP 1 : Culturing plant pollen

Dehisced anthers were detached from lily flowers (*Lilium longiflorum*) or pine trees and their pollen grains were collected and then stored in a deep freezer at -70°C until use. 0.5~5 g of the pollen grains were suspended in 200 mL of PGM and incubated at 25~30°C for 2~24 hours in the dark.

5 In the present invention, plant pollen is not limited to specific plant pollens, but all kinds of plant pollen can be used, including *Pinaceae*, *Ginkgoaceae*, as well as annual plants such as lily, cotton and tobacco.

In the present invention, the pollen growth medium (PGM) was preferably composed of 5~10% sucrose, 0.5~3 mM $\text{Ca}(\text{NO}_3)_2$, 50~300 μM
10 H_3BO_3 , 0.001~5 mM KNO_3 and 0.1~10 mM KH_2PO_4 , more preferably 7% sucrose, 1.27 mM $\text{Ca}(\text{NO}_3)_2$, 162 μM H_3BO_3 , 0.99 mM KNO_3 and 3 mM KH_2PO_4 .

STEP 2 : Construction of recombinant plasmid

15 A recombinant plasmid was constructed from the target gene by inserting to an *Agrobacterium* vector.

According to the present invention, ureB gene from *Helicobacter pylori* and tPA gene from humans were used for the pollen transformation.

In order to produce protein encoded by ureB gene in accordance with the
20 present invention, ureB gene was inserted into pBI121 with GUS reporter gene to obtain pBIUreB, which is the recombinant plasmid for *Agrobacterium* transformation. To produce protein encoded by tPA (tissue plasminogen activator), the tPA gene was inserted into pBI121 with GUS reporter gene to obtain pBI/tPA or pSK/tPA, which is the recombinant plasmid for *Agrobacterium*
25 transformation.

The recombinant proteins produced by the method of the present invention comprise some proteins produced from plant pollen, including ureB and tPA proteins.

STEP 3 : Transformation

The recombinant plasmid prepared in step 2 was introduced to *Agrobacterium tumefaciens* using the freeze-thaw method, incubated and selected in LB media with antibiotics such as kanamycin or cefotaxime.

5

STEP 4 : Introduction of gene via vacuum infiltration

In accordance with the present invention, the introduction of the gene to the plant pollen using *Agrobacterium* was carried out via particle bombardment, vacuum infiltration and electroporation.

10

STEP 5 : Identifying the target gene

Genomic DNA was extracted from germinated pollens with the recombinant gene according to the method of CTAB (Draper J. et al., *Plant genetic transformation and gene expression: A laboratory manual*, p204-208, Blackwell Scientific Publication, 1988).

15

The PCR technique can be used to determine whether the target gene was correctly introduced. In addition, the expression of the gene can be confirmed by conventional molecular techniques such as Southern blotting, Northern blotting and Western blotting. Through the molecular techniques, the enzymatic activity of β -glucuronidase expressed from pBI121, as well as the expression of the gene, could be assayed by histochemical staining, which turns into a blue color by reacting with a substrate, X-gluc.

20

In accordance with the present invention, 10 hours to 7 days is required to express recombinant protein by transformation and grow the pollen with the target gene.

25

The present invention will be explained in greater detail through reference with the following examples. However, the following examples are provided only to illustrate the present invention, and it should be understood that

the scope of the present invention is not limited thereto.

BRIEF DESCRIPTION OF THE INVENTION

5 The objective, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a microscopic photograph showing elongation of lily pollen tubes

10 Fig. 2 shows maps of pBIUreB with ureB and pBI121 with GUS reporter gene

Fig. 3 shows the result of identifying ureB gene in pollen using the PCR technique

Fig. 4 is a microscopic photograph showing expression of GUS enzyme in pollen

15 Fig. 5 is a photograph showing expression of ureB mRNA in pollen

Fig. 6 shows expression of UreB protein in pollen

Fig. 7 is a photograph showing expression of tPA cDNA in *E. coli* treated with IPTG by SDS-PAGE

20 Fig. 8 shows kanamycin toxicity to growth of lily pollen and the result of histochemical staining

Fig. 9 shows a result of Southern hybridization with genomic DNA of pollen

Fig. 10 shows a result of Western blotting of pollen protein

25 BEST MODE FOR CARRYING OUT THE INVENTION

Example 1 : Expression of ureB gene using lily pollen

To obtain the pollen for the present invention, dehisced anthers were

detached from lily flowers (*Lilium longiflorum*) and their pollen grains were collected and then stored in a deep freezer at -70°C until use. 1 g of the pollen grains were suspended in 200 mL of PGM composed of 7% sucrose, 1.27 mM Ca(NO₃)₂, 162 µM H₃BO₃, 0.99 mM KNO₃ and 3 mM KH₂PO₄ and incubated at 5 27°C for 3 hours in the dark. The result of the elongation of lily pollen according to times in growth medium is represented in Fig. 1.

To observe the expression of the gene in pollen, two kinds of recombinant plasmid were prepared. One of them was pBI121 with GUS reporter gene (Clontech) and the other was pBIUreB harboring ureB gene from 10 *Helicobacter pylori* (Genbank accession number AF352376: SEQ. No. 3). It has also been reported that recombinant UreB protein has an efficacy as anti-cancer vaccine. Processes for constructing pBIUreB are as following:

Primer No. 1: 5'-ATC CTA GAA TGA AAA AGA TTA GCA-3'(SEQ. ID No. 1) and primer No. 2: 5'-GAG CTC CTA GAA AAT GCT AAA GAG- 15 3'(SEQ. ID No. 2) were synthesized. From pH808 harboring urease gene from *Helicobacter pylori* (Lee et al., *J. Biochem. Mol.* 31, 240, 1998), 1.7 kb ureB DNA fragment (SEQ. ID No. 5) was amplified by PCR. The ureB DNA fragment was fused to pT7 Blue T-vector (Novagene) to construct pTureB. The resulting 1.7 kb ureB DNA obtained from pTureB was excised by cutting 20 with *Xba* I and *Sac* I was inserted to GUS-DNA deleted pBI121 by cutting with *Xba* I and *Sac* I to obtain pBIUreB. The recombinant diagram was represented in Fig. 2.

To introduce the target gene via *Agrobacterium* transformation and vacuum infiltration, the plasmids pBI121 and pBIUreB were transformed into 25 *Agrobacterium tumefaciens* A136(ATCC 51350) using the freeze-thaw method, respectively. Following this, they were selected and incubated in LB media with kanamycin.

To introduce the gene, the transformed *Agrobacterium tumefaciens*

harboring pBI121 and pBIUreB, respectively, were mixed with pollen pre-incubated for 3 hours, and then vacuum infiltration was carried out. The transformed *Agrobacterium* was grown in LB liquid medium with 50 mg/L of kanamycin and streptomycin, respectively. The bacterial cells were collected by
5 centrifuging 1 mL of the culture solution at 10,000 \times g for 3 min. and suspended in the pollen growth medium, including 50 mg/mL of pollen. The suspended solution was placed in a vacuum container under vacuum condition (-80 Pa, for 20 min) and filtered by filter paper. The pollens were washed twice with PGM with 200 mg/L of cefotaxime. The collected pollen were grown in 10 mL of
10 PGM at 27°C for 20 hours.

Experimental example 1 : Identifying target gene

To identify the target gene, fully-grown pollen tubes were collected. Genomic DNA was extracted from the pollen and PCR was performed for 35
15 cycles of the following reaction using the genomic DNA as a template; 94°C 40 sec, 65°C 1 min. 30sec, 72°C 2 min. Primers of SEQ. No. 1 and SEQ. No. 2 were used to identify ureB DNA. The resulting DNA was identified in 1% agarose gel electrolysis.

As shown in Fig. 3, the 1.7 kb DNA fragment from the genomic DNA of
20 the transformed lily pollen was amplified and is not shown in the untransformed pollen and in pBI121 transformed pollen.

Experimental example 2 : Assay of enzymatic activity of GUS in pollen

To confirm the expression of the target gene from the transformed pollen
25 via vacuum infiltration, the enzymatic activity of β -glucuronidase expressed from pBI121 was measured by histochemical staining. In more detail, the fully-grown pollen were suspended in X-gluc solution(100 μ L of 100 mM NaPO₄, 50 μ L of X-gluc(1 mg/mL of stock solution) and 850 μ L of sterile water), placed for 3

hours in the dark until a blue color came into view and then observed under microscope. The results were represented in Fig. 4.

As shown in Fig. 4, the untransformed pollen showed pale blue and the transformed pollen harboring pBI121 showed dark blue, which can confirm the expression of GUS gene.

Experimental example 3 : Expression of UreB gene in pollen by Northern blotting.

To monitor the expression of ureB gene in the transformed pollen harboring pBIUreB, the production of ureB mRNA was investigated using Northern blotting. PTUreB was digested with *Xba* I and *Sac* I and the resulting 1.7 kb DNA fragment was used as a probe. Probe labeling was carried out according to the manual of the random primed DNA labeling kit (Reche Molecular Biochemicals). RNA of fully-grown pollen tubes was extracted in extraction buffer with guanidine isothiocyanate and was electrophoresced on 1% formaldehyde gel. The gel was transferred to a Nytran membrane (Schleicher & Schuell) and blotted with the probe. Hybridization was performed at 42°C for 24 hours in a buffer containing 5 x SSC, 0.1% N-laurylsarcosine, 0.02% SDS, 2% blocking agent and 30% formamide, and washed with buffer containing 0.5 x SSC and 0.5% SDS. After this, the results were monitored by X-ray autoradiography. The results are represented in Fig. 5.

As shown in Fig. 5, 1.7 kb ureB mRNA was not expressed in the untransformed pollen, in pollen transformed by *Agrobacterium* and in pollen harboring pBI121, but was expressed in pollen harboring pBIUreB.

Experimental example 4 : Expression of UreB gene in pollen by Western blotting

Pollen samples were ground using a mini-pestle and suspended in an extraction buffer (KPO₄ pH 7.6, 2 mM PMSF and 10 mM EDTA). The pollen

lysates were centrifuged at $13,000 \times g$ for 10 min and the supernatant was collected. According to Lowry's method, total protein was measured quantitatively. 30 μg of protein was used for Western blotting assay. The extracted protein was electrophoresced on SDS- 8% polyacrylamide gel and the gel was transferred to Hybond -P-membrane (Amersham) in a transfer solution (0.025 M Tris-HCl, pH 8.3, 0.15 M glycine and 20% SDS) for 3~4 hours at 300 mA followed with Western blotting. The primary antibody used a rabbit anti-*H. pylori* urease IgG and the second antibody used a goat anti-rabbit IgG HRP-conjugate. Results were monitored by the ECL detection system (Amersham) and represented in Fig. 6. 67 kDa UreB protein was used as a control of recombinant ureases from *Helicobacter pylori*.

As shown in Fig. 6, a band in the same position as that of 67 kDa UreB protein of the control was not shown in untransformed pollen, in pollen infected by *Agrobacterium* and in pollen harboring pBI121, whereas UreB protein was expressed successfully in pollen harboring pBIUreB. Meanwhile, 0.05% of UreB protein of the total soluble protein could be estimated through densitometric analysis in comparison to Western blotting using the recombinant UreB protein from bacteria. The yield of UreB protein was calculated in 50 μg UreB protein per 1 g of pollen.

20

Example 2 : Expression of tPA gene using plant pollen

To produce a recombinant protein encoded by the target gene employing the lily pollen according to the present invention, tissue plasminogen activator (tPA) from human was expressed in transformed plant pollen.

25 According to the same method described in Example 1, lily pollen grains were collected and suspended in PGM composed of 7% sucrose, 1.8 mM $Ca(NO_3)_2$ and 1.6 mM H_3BO_3 , and incubated at $27^\circ C$ for 16 hours in the dark.

For transformation, recombinant pBI/tPA and pSK/tPA constructs were

constructed through the combination of pBI121 with tPA from human. The process for making the constructs are as follows: the primer No. 3 (sense) was designed to have *Xba* I restriction site fused to the start codon of tPA nucleotide sequence, 5'-AATCTAGACATGGATGCAATGAAGA-3' and the primer No. 4 (antisense) was designed to contain the stop codon followed by *Sac*I site, 5'-ATGATCTCTGGTCACGGTCGCATGTT-3'; From pETPFR(ATCC #40403) harboring tPA gene, tPA was amplified using tPA specific primers by PCR techniques. Polymerase Chain Reaction (PCR) was performed for 30 cycles of the following reaction: 94°C 30 sec, 53°C 30 sec, 72°C 2 min. The resulting 1.7 kb DNA fragment (SEQ. No. 6) was ligated to pT7BlueR (Novagen) plasmid to obtain the recombinant pT/tPA. From it, the tPA DNA was excised by cutting with *Xba*I and *Sac*I to be fused to pBluescript II SK (Stratagene) and to GUS-DNA deleted pBI121 (Clontech) by digesting with *Xba*I and *Sac*I to obtain pSK/tPA and pBI/tPA (not shown). After this, two kinds of recombinant plasmid were introduced into *E. coli* DH5 α and *E. coli* XL-1 Blue. And then, it was identified that the sequence inserted in the resulted 1.7 kb DNA fragment was tPA full length sequence.

Introduction of the recombinant plasmid pBI121 and pBI/tPA or pSK/tPA constructs into *Agrobacterium* and induction of target gene via vacuum infiltration were performed according to the method described in Example 1. Also, *Agrobacterium tumefaciens* LBA4404 transformed with the recombinant plasmid were grown in LB medium with kanamycin and cefotaxime. Concentration of kanamycin and cefotaxime and incubation condition of the *Agrobacterium tumefaciens* LBA4404 transformed was carried out in the same manner described in Example 1.

Experimental example 1 : Expression of tPA in *E. coli*

To confirm whether the 1.7 kb DNA fragment from the recombinant

plasmid constructed in Example 2 encodes tPA full length, *E. coli* XL-1BlueR cells transformed with the pSK/tPA were grown to the early log phase ($OD_{600} = 0.5$) in LB medium in shaker flasks and then treated with IPTG to a final concentration of 2 mM for further cultivation. The bacterial cells were collected
5 by centrifugation at $12,000 \times g$ for 5 min at $4^{\circ}C$ and suspended in extraction buffer (50 mM Tris-Cl, pH 7.5, 5 mM EDTA, 0.1% Tween 80, 2 mM PMSF) for disruption for three times in 2 min of sonication on ice. From the cell lysates, the inclusion body was collected by removing the supernatant following centrifugation at $12,000 \times g$ for 20 min at $4^{\circ}C$, and then used for SDS-PAGE and
10 immuno-blotting. The LacZ-fused tPA was calculated to be a 66 kDa protein (30 amino acids from the LacZ- α plus 562 amino acids of 62,900 Da tPA protein), approximately. Human tPA standard was paralleled to show its slightly larger size than the induced protein band. Fig. 7A represents a result of SDS-PAGE of inclusion bodies from IPTG-treated *E. coli* harboring pSK/tPA for 0 ~ 5 hrs,
15 wherein M represents protein size marker, C represents expression of tPA cDNA from pBluescript SK II transformed. Fig. 7B represents a result of Western blotting of the membrane-transferred bacterial proteins.

As shown in Fig. 7, although not strong, the induced synthesis of the 66 kDa protein evidently appeared at the one-hour point of the IPTG treatment and
20 continued to several hours examined in this experiment. From this result, the induced protein was almost expected to be the fused tPA, but its further confirmation was carried out by Western blotting.

Experimental example 2 : Assay of enzymatic activity of GUS in pollen by 25 histochemical staining

In order to demonstrate the expression of target gene from the pollen, activity of GUS enzyme was assayed by histochemical staining according to experimental example 1 of Example 1. Fig. 8A represents kanamycin toxicity to

elongation of pollen tubes, wherein a, b, c, d, e and f represent 0, 50, 100, 125, 150 and 200 mg/L of kanamycin added in the pollen growth media, respectively. Fig. 8B represents a result of histochemical staining of pollen tubes of the untransformed (con), pBI121 transformed (tra) and a magnified feature of the tra (mag).

As shown in Fig. 8, most of the transformed pollen grown with or without kanamycin was shown to be similarly demonstrating the GUS activity, implying that most of the pollen population successfully transformed by vacuum-infiltration.

Experimental example 3 : Expression of tPA in pollen by Southern blotting

Fully-grown pollen tubes were washed several times with sterile distilled water, ground to a fine powder in the presence of liquid nitrogen using mortar and pestle, and used for extracting genomic DNA according to the CTAB/chloroform method (Taylor et al., 1993). Genomic DNA digested with *Xba*I and *Sac*I was electrophoresced on a 0.8% agarose gel and transferred to a nylon membrane (Hybond N, Amersham) for hybridization with 1.7 kb tPA DNA. Probe labeling was done according to the ECL random prime system (Pharmacia). Hybridization was performed at 60°C for 24 hrs in buffer containing 5x SSC, 0.1% SDS, 5% dextran sulfate, 100 µg/mL denatured sheared salmon sperm DNA and 5% Pharmacia liquid block. Results were monitored by ECL detection system (Pharmacia) following anti-fluorescein-HRP reaction.

As shown in Fig. 7, 1.7 kb DNA fragment was evident from the transformed, but none from the untransformed, strongly indicating tPA DNA integration into the pollen chromosomal DNA. Total soluble proteins estimated about 12% of the pollen weight were extracted for SDS-12% PAGE followed

with Western blotting.

As shown in Fig. 9, protein bands in recognition to the tPA mAb could be observed at the location nearly identical to the standard tPA and expected to have a similar extent of glycosylation in mass in lily pollen. Approximately
5 0.05% of tPA protein of the total soluble proteins could be estimated through densitometric analysis in comparison to the standard tPA.

Experimental example 4 : Expression of tPA in pollen by Western blotting

10 Expression of tPA recombinant protein was carried out by Western blotting. Pollen samples were ground in liquid nitrogen using a mortar and pestle and suspended in extraction buffer (50 mM Tris-Cl, pH 7.5, 5 mM EDTA, 0.1% Tween 80, 2 mM PMSF). The pollen lysates were centrifuged at $12,000 \times g$ for 15 min at 4°C and the supernatant was used for SDS-12% polyacrylamide gel
15 electrophoresis and Western blotting, according to the standard technique (Sambrook and Russell, 2001). The tPA protein was detected using mAb-tPA (Biodesign, ME) and Western blue stabilized substrate for alkaline phosphatase (Promega, WI). Single-chain tPA from human melanoma cell culture (Sigma, Mo) was used as a standard. Fig. 10 represents a result of Western blotting of
20 pollen proteins, wherein M represents the protein size marker, Con represents pBI121-transformed pollen, S30 and S60 represent the standard tPA 30 ng and 60 ng, respectively and Tra represents pBI/tPA transformed.

As shown in Fig. 10, the transferred 66 kDa protein bands onto PVDF membrane could be detected as colored ones at their corresponding locations in
25 recognition to mAb against human tPA. Taken these results together, the 1.7 kb PCR product amplified from pETPFR plasmid could be identified for its encoded tPA protein in size and immune reaction.

INDUSTRIAL APPLICABILITY

As described before, the present invention comprises the use of plant pollen as a host for producing recombinant protein, which makes it possible to easily produce the recombinant protein at lower prices in comparison to
5 production systems employing animal cell lines due to the absence of complicated costs in cell proliferation, maintenance of cellular genetic stability and operation of bioreactor.

Also, the present invention can directly produce recombinant protein in a pollen growth culture from a few hours to maximum of several days in a simple
10 liquid medium supplemented with 3~4 kinds of chemicals, including sugar. Therefore, the present invention is very useful in medical and genetic engineering industries.

WHAT IS CLAIMED IS

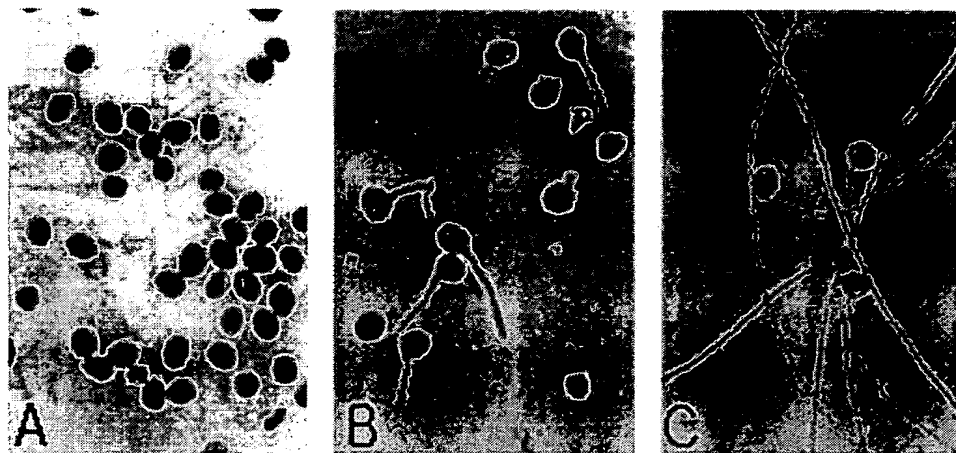
1. A method for producing recombinant protein using plant pollen
5 comprising the steps of:
isolating pollen from plant;
culturing the pollen in a pollen growth medium;
introducing a target gene into *Agrobacterium* vector to be transformed;
infecting the transformed *Agrobacterium* into the cultured plant pollen;
10 and
growing the pollen with the gene in a pollen growth medium to obtain the
resulting recombinant protein.
2. The method of production as set forth in claim 1, wherein the plant pollen
15 is isolated from *Lilium longiflorum* or *Pinaceae*.
3. The method of production as set forth in claim 1, wherein the pollen
growth medium is composed of 5~10% sucrose, 0.5~3 mM $\text{Ca}(\text{NO}_3)_2$,
50~300 μM H_2BO_3 , 0.001~5 mM KNO_3 and 0.1~10 mM KH_2PO_4 .
20
4. The method of production as set forth in claim 1, wherein the gene is
introduced into *Agrobacterium* by a method selected from the group
consisting of particle bombardment, vacuum infiltration and
electroporation.
- 25 5. The method of production as set forth in claim 1, wherein the target gene
is UreB gene from *Helicobacter pylori* and tissue plasminogen activator
(tPA) from humans.

ABSTRACT

The invention relates to a method for producing recombinant protein using pollen. More specifically, the invention relates to a method for producing recombinant protein by introducing a recombinant gene into pollen using
5 *Agrobacterium tumefaciens* via vacuum infiltration and then inducing pollen tube growth, which makes it possible to scale-up the production of recombinant protein within a short period and at lower production costs.

1/9

Fig. 1

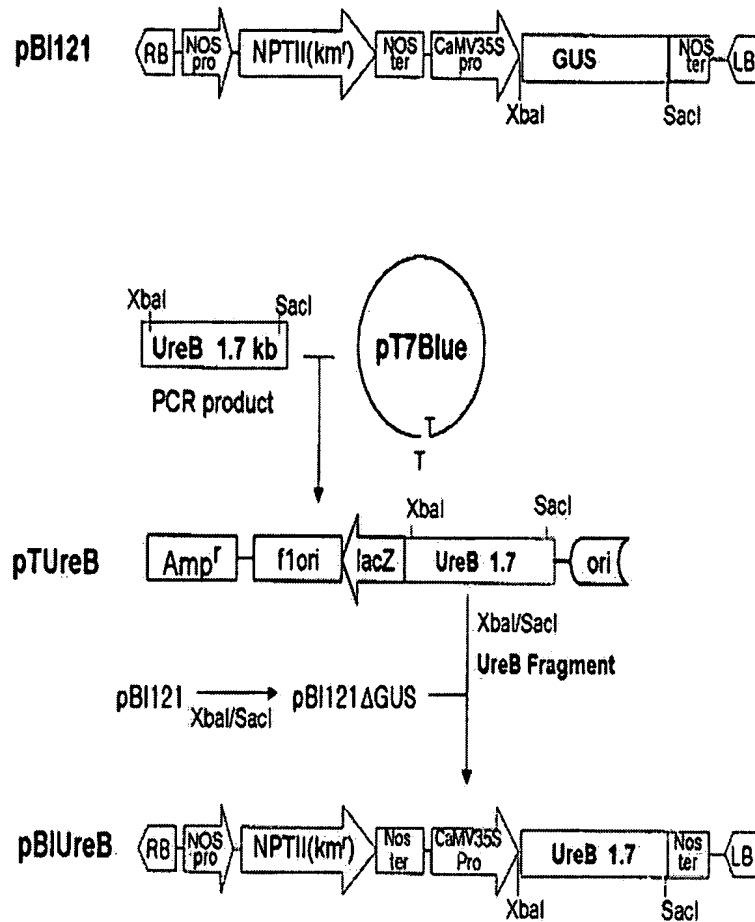


**[Note] Microscopic photograph of lily pollens on 0 hr(A), 8 hr(B),
16 hr(C) after culturing in pollen growth medium**

10/500447

2/9

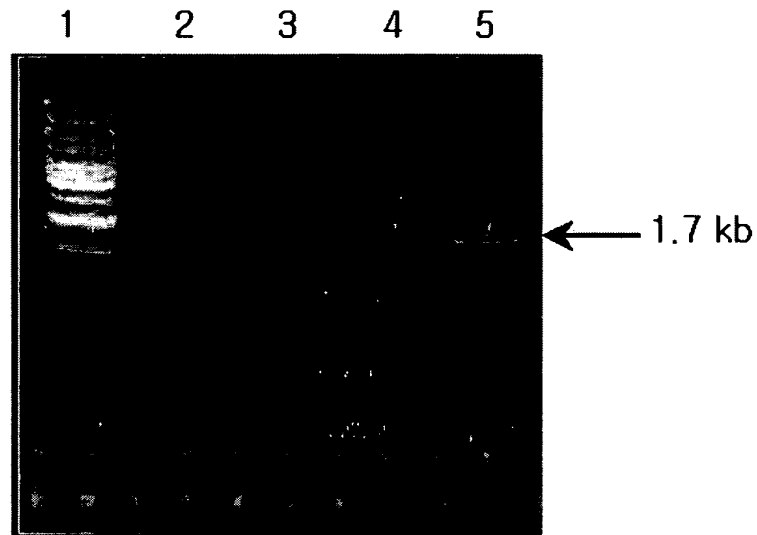
Fig. 2



[Note] Constructive diagram of pBI121 reporter gene and pBIUreB gene

3/9

Fig. 3

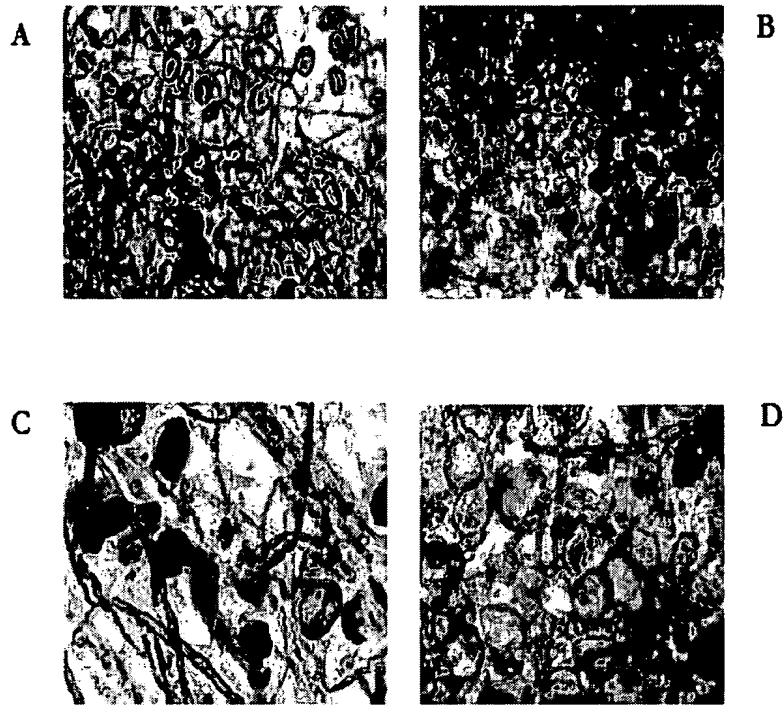


[Note] Identification of inserted gene by PCR

1. DNA size marker
2. Untransformed lily pollen.
3. *Agrobacterium tumefaciens* A136-transformed pollen
4. Pollen harboring pBI121
5. Pollen harboring pBIUreB

4/9

Fig. 4



[Note] Assay of enzymatic activity of GUS by histochemical staining

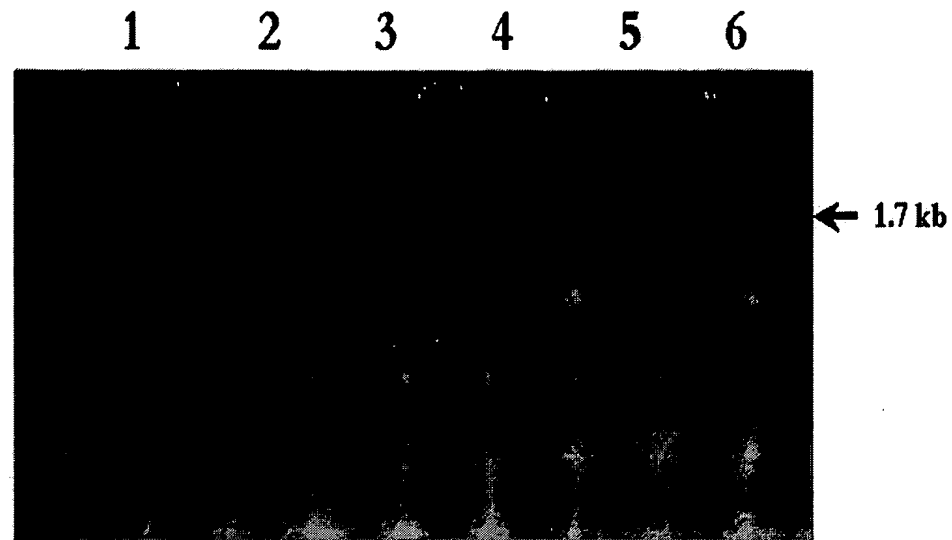
A : Untransformed lily pollens

B : lily pollens harboring GUS reporter gene

C, D : Enlarged photograph of B

5/9

Fig. 5



[Note] Expression of ureB mRNA in pollen harboring pBIUreB

1. RNA size marker

2. UreB DNA

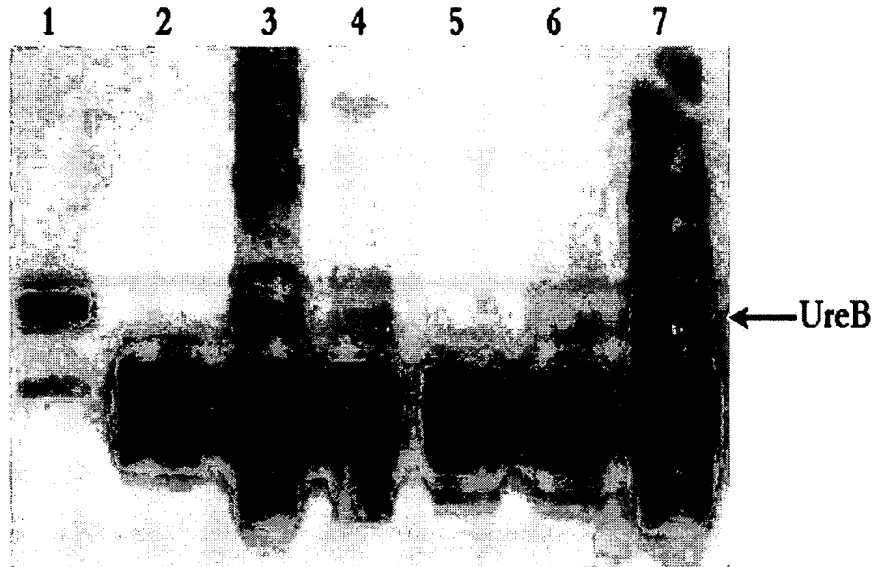
3. *Agrobacterium tumefaciens* A136-transformed pollen

4. Lily pollen harboring pBI121

5. Lily pollen harboring pBIUreB

6/9

Fig. 6

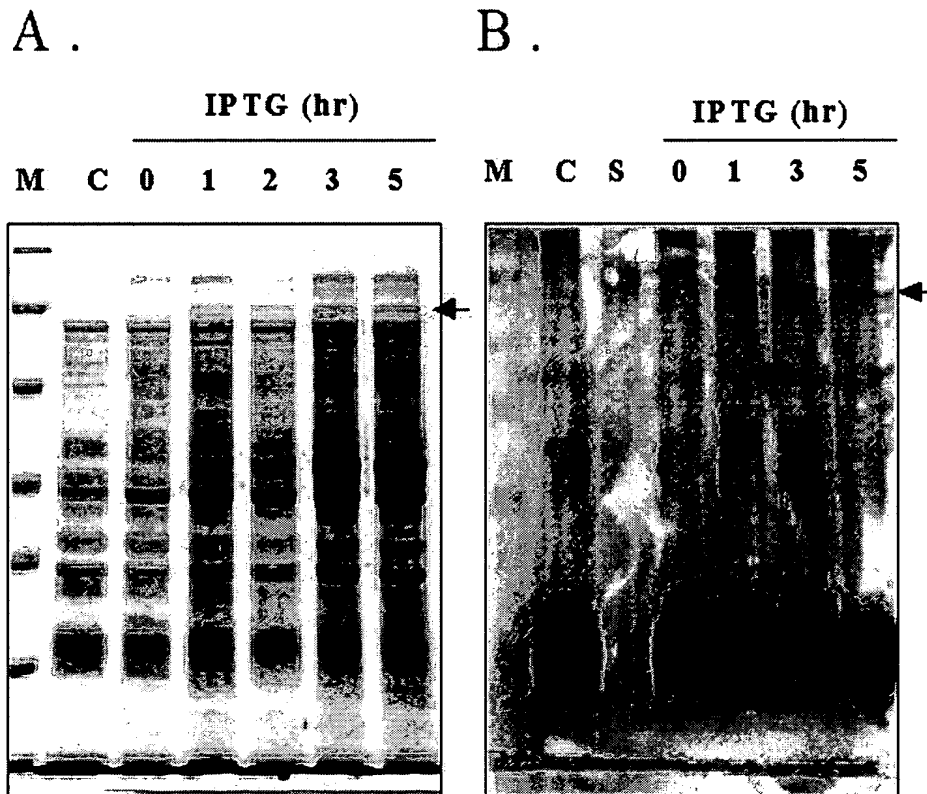


[Note] Production of UreB protein in pollen

1. Recombinant UreB protein of *Helicobacter pylori*
2. *Agrobacterium tumefaciens* A136
3. Untransformed lily pollen
4. *Agrobacterium tumefaciens* A136-transformed pollen
5. Lily pollen harboring pBI121
6. Lily pollen harboring pBIUreB(on 0hr after transformation)
7. Lily pollen harboring pBIUreB(on 24hr after transformation)

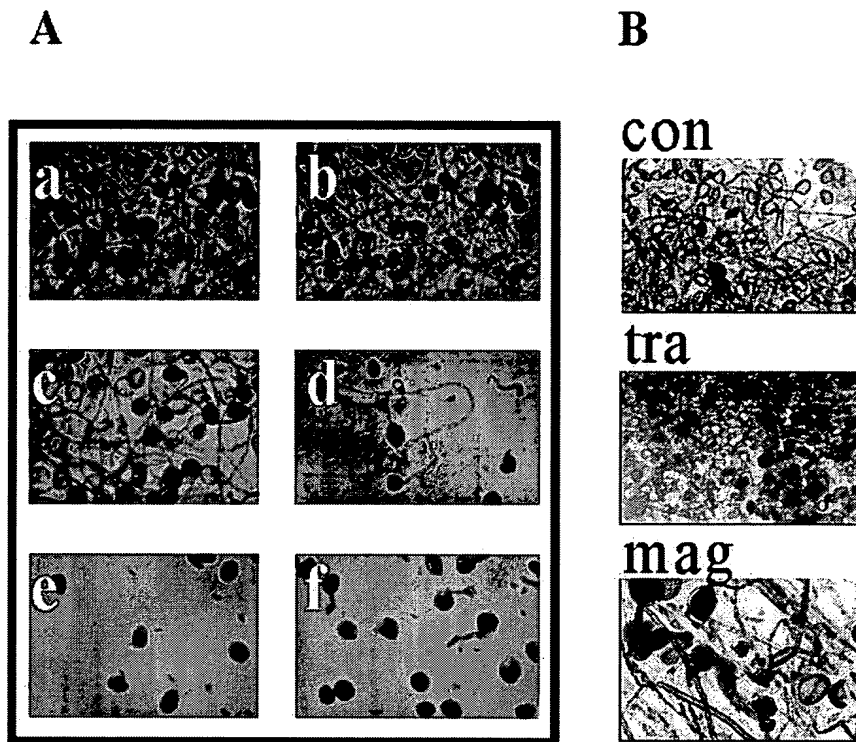
7/9

Fig. 7



8/9

Fig. 8



9/9

Fig. 9

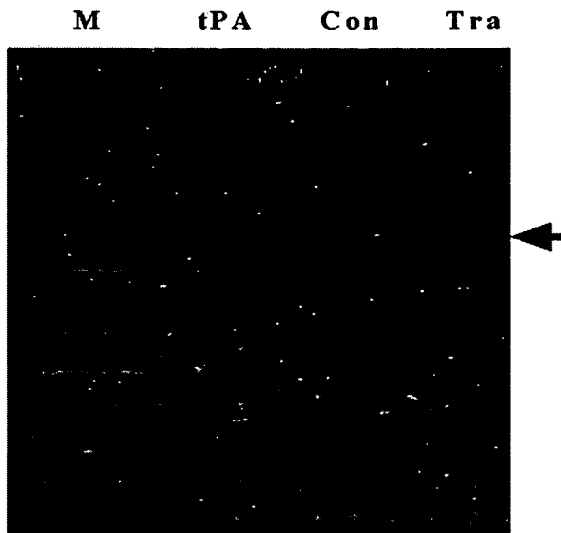
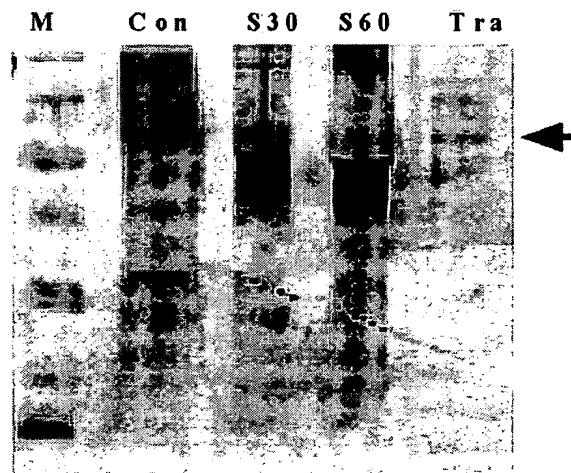


Fig. 10



SEQUENCE LISTING

0 / 5 0 0 4 4 7
DT04 Rec'd PCT/PTO 30 JUN 2004

<110> PARK, Hee-Sung

<120> Method for producing a recombinant protein using pollen

<130> YL0P040518US/PCT

<150> KR 2001-71712

<151> 2001-11-19

<160> 6

<170> PatentIn version 3.2

<210> 1

<211> 24

<212> DNA

<213> Oligonucleotide for amplification of urease B gene

<400> 1

atcctagaat gaaaaagatt agca 24

<210> 2

<211> 24

<212> DNA

<213> Oligonucleotide for amplification of urease B gene

<400> 2

gagctcctag aaaatgctaa agag 24

<210> 3

<211> 25

<212> DNA

<213> Oligonucleotide for amplification of tissue plasminogen activator

<400> 3

aatctagaca tggatgcaat gaaga 25

<210> 4

<211> 26

<212> DNA

<213> Oligonucleotide for amplification of tissue plasminogen activator

<400> 4

atgatctctg gtcacggtcg catgtt 26

<210> 5

<211> 1710

<212> DNA

<213> Helicobacter pylori

<400> 5

atgaaaaaga ttagcagaaa agaatatgtt tctatgtatg gccctactac aggcgataaa 60

gtgagattgg gcgatacaga cttgatcgct gaagtagaac atgactacac catttatggc 120

gaagagctta aattcggcgg tggtaaaacc ctaagagaag gcatgagcca atctaacaac 180

cctagcaaag aagaactgga tctaatacat actaacgctt taatcgtgga ttacaccggt 240

atttataaag cgcatattgg tattaaagat ggcaaaatcg ctggcattgg taaaggcggt 300

aacaagaca tgcaagatgg cgttaaaaac aatcttagcg tgggtcctgc tactgaagcc 360

ttagccggtg aaggtttgat cgtaactgct ggtggtattg acacacacat ccacttcac 420

tcccccaac aaatccctac agcttttgca agcgggtgaa caacgatgat tgggtggcga 480

actggccctg ctgatggcac taacgcaacc actatcactc caggtagaag aaatttaaa 540

tggatgctca gagcggcaga agaataattct atgaacttaa gtttcttagc taaaggtaac 600

gcttctaacg atgcaagcgt agccgatcaa attgaagccg gtgcgattgg ctttaaaatc	660
cacgaagact ggggcaccac tccttctgca atcaatcatg cgttagatgt tgcggacaaa	720
tacgatgtgc aagtgcgtat ccacacagac actttgaatg aagccggttg tgtagaagac	780
actatggcag ccattgccgg acgcactatg cacactttcc acactgaagg cgctgggtggc	840
ggacacgctc ctgatattat taaagtagct ggtgaacaca acattctgcc cgcttccact	900
aaccccaacta tccctttcac tgtgaatata gaagcagaac acatggacat gcttatggtg	960
tgccaccact tggataaaag cattaagaa gatgttcagt tcgctgattc aaggatccgc	1020
cctcaaaacta ttgcggctga agacactttg catgacatgg ggattttctc aatcaccagt	1080
tctgactctc aagctatggg tcgtgtgggt gaagttatca ccagaacttg gcaaacagct	1140
gacaaaaaca aaaaagaatt tggccgcttg aaagaagaaa aaggcgataa cgacaacttc	1200
aggatcaaac gctacttgtc taaatacacc attaacccag cgatcgctca tgggattagc	1260
gagtatgtag gttctgtaga agtgggcaaa gtggctgact tgggtttgtg gagtcccgca	1320
ttctttggcg tgaacccaa catgatcatc aaaggcggat tcattgcatt gagtcaaag	1380
ggtgatgca acgcttctat ccctacccca caaccggttt attatagaga aatgttcgct	1440
catcatggta aagctaaata cgatgcaaac atcacttttg tgtctcaagc ggcttatgac	1500
aaaggcatta aagaagaatt agggcttgaa aggcaagtgt tgcgggtaaa aaattgcaga	1560
aacatcacta aaaaagacat gcaattcaac gacactaccg ctcacattga agtcaatcct	1620
gaaacttacc atgtgttcgt ggatggcaaa gaagtaactt ctaaacaccg caataaagt	1680
agcttggcac aactctttag cattttctag	1710

<210> 6
 <211> 2280
 <212> DNA
 <213> Homo sapiens

<400> 6	
ggagtccagg gctggagaga aaacctctgc gaggaaggagg aaggagcaag ccgtgaattt	60
aaggacgct gtgaagcaat catggatgca atgaagagag ggctctgctg tgtgctgctg	120
ctgtgtggag cagtcttcgt ttgcccagc caggaaatcc atgcccgaat cagaagagga	180
gccagatctt accaagtgat ctgcagagat gaaaaaacgc agatgatata ccagcaacat	240
cagtcatggc tgcgccctgt gctcagaagc aaccgggttg aatattgctg gtgcaacagt	300
ggcagggcac agtgccactc agtgccctgc aaaagtigca gcgagccaag gtgtttcaac	360
gggggcacct gccagcaggc cctgtacttc tcagatttcg tgtgccagt ccccgaaagga	420
tttctgtgga agtgctgtga aatagatacc agggccacgt gctacgagga ccagggcatc	480
agctacaggg gcacgtggag cacagcggag agtggcgccg agtgaccaa ctggaacagc	540
agcggttgg ccagaagcc ctacagcggg cggaggccag atgcatcag gctgggcctg	600
gggaaccaca actactgcag aaaccagat cgagactcaa agccctggtg ctacgtcttt	660
aaggcgggga agtacagctc agagtctgc agcaccctg cctgctctga gggaaacagt	720
gactgctact ttgggaatgg gtcagcctac cgtggcacgc acagcctcac cgagtcgggt	780
gcctcctgcc tccgtggaa ttccatgac ctgataggca aggtttacac agcacagaac	840
cccagtggcc aggcactggg cctgggcaaa cataattact gccggaatcc tgatggggat	900

gccaaagccct ggtgccacgt gctgaagaac cgcaggctga cgtgggagta ctgtgatgtg 960
 ccctcctgct ccacctgcgg cctgagacag tacagccagc ctacagtttcg catcaaagga 1020
 gggctcttcg ccgacatcgc ctcccacccc tggcaggctg ccatctttgc caagcacagg 1080
 aggtcgcccc gagagcgggt cctgtgcggg ggcatactca tcagctcctg ctggattctc 1140
 tctgccgccc actgcttcca ggagagggtt ccgccccacc acctgacggt gatcttgggc 1200
 agaacatacc ggggtgtccc tggcgaggag ggcagaaat ttgaagtcga aaaatacatt 1260
 gtccataagg aattcgatga tgacacttac gacaatgaca ttgcgctgct gcagctgaaa 1320
 tggattcgt ccgcctgtgc ccaggagagc agcgtggtcc gcactgtgtg ccttcccccg 1380
 gcggacctgc agctgccgga ctggacggag tgtgagctct ccggtacgg caagcatgag 1440
 gccttgtctc ctttctattc ggagcggctg aaggaggctc atgtcagact gtacccatcc 1500
 agccgctgca catcacaaca ttacttaac agaacagtca ccgacaacat gctgtgtgct 1560
 ggagacactc ggagcggcgg gccccaggca aacttgcacg acgcctgccg gggcgattcg 1620
 ggaggccccc tgggtgtgtc gaacgatggc cgcattgactt tggggggcat catcagctgg 1680
 ggcttgggct gtggacagaa ggaatgtccc ggtgtgtaca ccaaggttac caactaccta 1740
 gactggattc gtgacaacat gcgaccgtga ccaggaacac ccgactcctc aaaagcaaat 1800
 gagatccgc ctcttcttct tcagaagaca ctgcaaaggc gcagtgttc tctacagact 1860
 tctccagacc caccacaccg cagaagcggg acgagaccct acaggagagg gaagagtga 1920
 ttttcccaga tacttcccat ttggaagtt ttcaggactt ggtctgattt caggatactc 1980
 tgtcagatgg gaagacatga atgcacacta gccctctccg gaatgcctcc tccctgggca 2040
 gaaagtggcc atgccaccct gttttcagct aaagcccaac ctctgacct gtcaccgtga 2100
 gcagctttgg aaacaggacc acaaaaatga aagcatgtct caatagtaaa agataacaag 2160
 atctttcagg aaagacggat tgcattagaa atagacagta tatttatagt cacaagagcc 2220
 cagcagggcc tcaaagttgg ggcaggctgg ctggcccgctc atgttctca aaagaccct 2280

A. CLASSIFICATION OF SUBJECT MATTER

• IPC7 C07K 14/415

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C07K 14/415

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, MEDLINE, DELPHION

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5066594 A (DNA Plant Technology) 19 NOVEMBER 1991 see the whole document	1 - 5
A	US 5929300 A (THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY OF AGRICULTURE) 27 JULY 1999 see the whole document	3, 4
A	SOWA et al. 'Biochemical changes during pollen germination measured in vivo by infrared spectroscopy' In: 'Plant Science, 1995, Vol.105, pp23-30 see the whole document	1 - 5
P. A	ROKOCZY-TROJANOWSKA 'Alternative methods of plant transformation - a short review' In: Cell Mol Biol Lett, 2002, Vol.7, No.3, pp849-858 see abstract	4

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family


Date of the actual completion of the international search

12 MARCH 2003 (12.03.2003)

Date of mailing of the international search report

13 MARCH 2003 (13.03.2003)

Name and mailing address of the ISA/KR

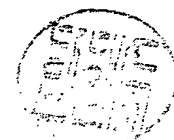

 Korean Intellectual Property Office
 920 Dunsan-dong, Seo-gu, Daejeon 302-701,
 Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer:

HAN, Hyun Sook

Telephone No. 82-42-481-5596



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

KR02/02154

Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

US 5066594 A

19.11.91

NONE

US 5929300 A

27.07.99

WO 9903326 A1

28.01.99

EP 0996328 A1

03.05.00

CN 1263434 T

16.08.00

AU 8400598 A1

10.02.99

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☒ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.